IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kazuhisa HATAKEYAMA

Application No.: 09/576,715 Group Art Unit: 1655

Filing Date; 05/23/00 Examiner: FORMAN BJ

Title: METHOD FOR GENE ANALYSIS

DECLARATION UNDER 37 CFR 1.132

Assistant Commissioner for Patent, Washington, D.C.

Sir:

I, Kazuhisa Hatakeyama, a citizen of Japan and resident at 1-11-3-206, Amimachi-chuo, Inashiki-gun, Ibaraki-ken, 300-0332, Japan, declares as follows:

That in March 1988, I graduated from the University of Tokyo, Faculty of Agriculture, Department of Agricultural Chemistry, receiving the degree of Master of Agriculture.

That in April 1988, I joined Tsukuba Research Center, Mitsubishi Petrochemical Co. Ltd. (now Mitsubishi Chemical Corporation) as a researcher and I am engaged mainly in research in molecular microbiology.

That my achievements include the following publications:

1. Journal of Industrial Microbiology, 5, 1990, 159-166

"Electrotransformation of intact cells of

Brevibacterium flavum MJ233"

2. DNA Sequence, 4, 1993, 87-93

"Analysis of the biotin biosynthesis pathway in coryneform bacteria: Cloning and sequencing of the *bioB* gene from *Brevibacterium flavum*"

3. DNA Sequence, 4, 1993, 177-184

"Genomic organization of the biotin biosynthetic genes of coryneform bacteria: Cloning and sequencing of the *bioA-bioD* genes from *Brevibacterium flavum*"

4. Bioscience, Biotechnology and Biochemistry, 57, 1993, 2036-2038

"Replacement recombination in coryneform bacteria: High efficiency integration requirement for non-methylated plasmid DNA"

5. Journal of Biological Chemistry, 272, 1997, 14727-14732

"Novel genes encoding 2-aminophenol 1, 6-dioxygenase from *Pseudomonas* species AP-3 growing on 2-aminophenol and catalytic properties of the purified enzyme"

6. Biochemical and Biophysical Research Communications, 236, 1997, 383-388

"Cloning and sequencing, and characterization of the *ftsZ* gene from coryneform bacteria"

7. Methods in Enzymology, 279, 1997, 339-348

"Analysis of biotin biosynthesis pathway in

coryneform bacteria: *Brevibacterium flavum*"

8. Bioscience, Biotechnology and Biochemistry, 64, 2000, 1477-1485

"Analysis of oxidation sensitivities of maleate *cis-trans* isomerase from *Serratia marcescens*"

9. Bioscience, Biotechnology and Biochemistry, 64, 2000, 569-576

"Molecular analysis of maleate *cis-trans* isomerase from thermophilic bacteria"

10. Gene Medicine, 4, 2000, 58-62

"SNP detection by DNA chip"

11. Nippon Rinsho, 57, 1999, 215-223

"High-throughput gene analysis with DNA array-from sequencing to expression monitoring"

12. Biochemical and Biophysical Research Communications, 239, 1997, 74-79

"Gene cloning and characterization of maleate *cis-trans* isomerase from *Alcaligenes faecalis*"

In order to show the effects of the present invention, the following experiments were conducted under my direction and supervision.

Experiments

I. DNA annealing assay by gel-electrophoresis

In order to analyze effect of Sso7d protein on annealing activity of complementary single-stranded DNA, annealing assay was performed by using M13mp18 circular ss DNA (produced by Takara Shuzo Co.) and the probe nucleic acid (oligonucleotide probe) (17 mer) which was complementary to M13mp18 circular ss DNA, and whose end was fluorescent labeled with FITC. The oligonucleotide probes synthesized by using the known method in accordance with the sequence described in table 1 were used. To compare annealing efficiency, annealing assay samples of each oligonucleotide probe with or without Sso7d protein were prepared.

Table 1

Name of probe	Nucleotide sequence	Note
M13FUP17R	5'-FITCgtaaaacgacggccagt-3'	Completely matched probe
M13FUP17Rmpt	5'-FITCgtaaaacgtcggccagt-3'	internal one base-mismatched probe
M13FUP17Rmp23	5'-FITCgtaaaacgacggccatc-3'	terminal two base-mismatched probe
M13FUP17Rmp3	5'-FITCgtaaaacgacggccaga-3'	terminal one base-mismatched probe

To 10 μl of annealing buffer (buffer composition: 20 mM Tris buffer, pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 100 μg/μl BSA (all showed in the final concentration)), were 0.2 pmol of M13mp18 ss DNA and 0.2 pmol of each end-labeled oligonucleotide probe added to prepare the annealing assay samples of each oligonucleotide probe with or without Sso7d protein. Then they were mixed and incubated at 65 C° for

3 minutes to react. To the reaction mixture were 1 μl of reaction stopping agent (10 % SDS, 0.25 % bromophenol blue, 0.25 % bromocyanol blue) added to quench the reaction. Then the resulting mixture was applied to 1.5 % (w/v) of agarose gel to perform electrophoresis (100 V for 30 minutes) at room temperature by using Tris-acetic acid buffer. After electrophoresis, fluorescence of the gel was detected and then quantified by Fluoro Imager SI (produced by Molecular Probes Inc.). 530DF30 was used as a filter for detecting.

In the method, DNA sequence used, buffer composition, electrophoresis procedure and the like were similar to that of the method described in Guagliardi et al.(Journal of Molecular Biology, 1997, 267: 841-848) except the reaction temperature being 65 C° not 60 C° and signal detecting method being FITC fluorescent detection not RI method.

As a result, annealing was barely detected for the annealing sample without Sso7d protein, while annealing was detected for the annealing sample with Sso7d protein and completely matched probe. In the case when the Sso7d protein was added, and the S/N ratio was calculated by using background value, a result was about 2.2 (10476/4720). The difference of the signal intensity between the case of using the completely matched probe and the case of using internal one base-mismatched probe was a little less than double.

Figure 1 shows the picture of the resulting fluorescent gel and Table 2 shows quantitative result. The numbers described in Table 2 correspond to the lane numbers of the

picture described in Figure 1.

Table 2

No.	Probe	Signal intensity	Note
Without Sso7d (-Sso7d)			
1	M13FUP17R	6255	completely matched probe
2	M13FUP17Rmpt	4463	internal one base-mismatched probe
3	M13FUP17Rmp23	4840	terminal two base-mismatched probe
4	M13FUP17Rmp3	5977	terminal one base-mismatched probe
With Sso7d (+Sso7d)			
5	M13FUP17R	10476	completely matched probe
6	M13FUP17Rmpt	5467	internal one base-mismatched probe
7	M13FUP17Rmp23	7122	terminal two base-mismatched probe
8	M13FUP17Rmp3	11063	terminal one base-mismatched probe
Background		4720	

II. DNA hybridization with DNA microarray

(1) Preparation of probe nucleic acids (oligonucleotide probes) and DNA samples

The oligonucleotides were synthesized by using a DNA synthesizer (apparatus name: Expedite 8909, manufactured by PerSeptive Biosystems Inc.). That is, the oligonucleotides (probes and samples) shown in the following Table 3 were synthesized in accordance with Nucleic Acid Synthesis System User's Guide of PerSeptive Biosystems Inc. by using a cycle in which the trityl groups were not removed, which were the

protective groups of the 5' ends. The synthesized oligonucleotides were purified by using Poros Oligo R3 (produced by PerSeptive Biosystems Inc.).

The oligonucleotides Nos. (i)-(x) and Nos. (4)-(7) of oligonucleotides shown in the following Table 3 are oligonucleotide probes. The oligonucleotide Nos. (xi) and (8) are DNA samples. The sequences of oligonucleotide Nos. (4)-(8) correspond to the sequences of SEQ ID Nos. (4)-(8) in SEQUENCE LISTING of the present application.

The 3′ ends of the oligonucleotides for immobilization were modified by using 3'-aminomodifier C3 (produced by Glen Research) to facilitate immobilization on a substrate. The 5′ ends of DNA samples were labeled by using Cy3 or Cy5 Amidite (produced by Amersham Pharmacia Biotech Inc.).

Table 3

No.		Nucleotide sequence	Note
(i)	BAR121T	5'-CTggTCATCgTggCCATCgCCZ-3'	
(ii)	BAR121C	5'-CTggTCATCgCggCCATCgCCZ-3'	
(iii)	BAR119T	5'-TggTCATCgTggCCATCgCZ-3'	
(iv)	BAR119C	5'-TggTCATCgCggCCATCgCZ-3'	
(v)	BAR117T	5'-ggTCATCgTggCCATCgZ-3'	
(vi)	BAR117C	5'-ggTCATCgCggCCATCgZ-3'	
(vii)	BAR115T	5'-gTCATCgTggCCATCZ-3'	
(viii)	BAR115C	5'-gTCATCgCggCCATCZ-3'	
(5)	BAR113T	5'-ATCgCCTggACTCZ-3'	
(4)	BAR113C	5'-ATCgCCCggACTCZ-3'	
(ix)	BAR113iT	5'-iiiiATCgCCTggACTCiiiiZ-3'	
(x)	BAR113iC	5'-iiiiATCgCCCggACTCiiiiZ-3'	
(7)	BAR111iT	5'-iiiiTCgCCTggACTiiiiZ-3'	
(6)	BAR111iC	5'-iiiiTCgCCCggACTiiiiZ-3'	
(xi)	TBARW26C3	5'-Cy3AgTCTCggAgTCCAggCgATggCCAC-3'	(26mer+Cy3)
(8)	TBARW26C5	5'-Cy5AgTCTCggAgTCCgggCgATggCCAC-3'	(26mer+Cy5)

(In the table, i represents inosine residue, z represents 3'-aminomodifier C3, Cy3 represents a fluorescent label by Cy3 and Cy5 represents a fluorescent label by Cy5.)

The above oligonucleotides were concentrated to dryness, and then suspended in a 0.5 M sodium hydrogencarbonate buffer (pH 8.4) for the oligonucleotide

probes Nos. (i)-(x) and Nos. (4)-(7), or TE buffer for the DNA samples Nos. (xi) and (8), and they were quantified based on absorbance at 260 nm and adjusted to 100 pmol/μl.

(2) Immobilization of oligonucleotide probes (preparation of DNA microarray)

Immobilization of the oligonucleotides prepared in the aforementioned step (1) to a substrate was performed as follows. First, solutions of the oligonucleotide probes were each spotted onto a Silylated Slide of TeleChem Inc. (microscope slide having aldehyde residues on its surface) using a GTMASS Stamping apparatus produced by Nippon Laser & Electronics Lab. Spots were made on a slide in the form of 4 columns from left to right, and 14 rows from top to down. The total spots are 56 per microscope slide. Each solution of the oligonucleotide probes and a 0.5 M sodium hydrogencarbonate buffer (pH 8.4) as blank were spotted two by two alternately. Each oligonucleotide probe used and immobilized points of spots are shown in Table 4.

Table 4

Row	Column	Probes	chain length	Cy3(top)	Cy5(lower)
1	4	BAR121T	21mer	completely matched with TBARW26C3	one base mismatched with TBARR26C5
2	4	BAR121T			
3	4	Blank			
4	4	Blank			
5	4	BAR121C	21mer	one base mismatched with TBARW26C3	completely matched with TBARR26C5
6	4	BAR121C			
7	4	Blank			
8	4	Blank			
9	4	BAR119T	19mer	completely matched with TBARW26C3	one base mismatched with TBARR26C5
10	4	BAR119T			
11	4	Blank			
12	4	Blank			
13	4	BAR119C	19mer	one base mismatched with TBARW26C3	completely matched with TBARR26C5
14	4	BAR119C			
1	3	Blank			
2	3	Blank			
3	3	BAR117T	17mer	completely matched with TBARW26C3	one base mismatched with TBARR26C5
4	3	BAR117T			
5	3	Blank			
6	3	Blank			
7	3	BAR117C	17mer	one base mismatched with TBARW26C3	completely matched with TBARR26C5
8	3	BAR117C			
9	3	Blank			
10	3	Blank			
11	3	BAR115T	15mer	completely matched with TBARW26C3	one base mismatched with TBARR26C5
12	3	BAR115T			
13	3	Blank			
14	3	Blank			
1	2	BAR115C	15mer	one base mismatched with TBARW26C3	completely matched with TBARR26C5
2	2	BAR115C			
3	2	Blank			
4	2	Blank			
5	2	BAR113T	13mer	completely matched with TBARW26C3	one base mismatched with TBARR26C5
6	2	BAR113T			
7	2	Blank			
8	2	Blank			
9	2	BAR113C	13mer	one base mismatched with TBARW26C3	completely matched with TBARR26C5
10	2	BAR113C			
11	2	Blank			
12	2	Blank			
13	2	BAR113iT	13mer + 4 inosine	completely matched with TBARW26C3	one base mismatched with TBARR26C5
14	2	BAR113iT			
1	1	Blank			
2	1	Blank			
3	1	BAR113iC	13mer + 4 inosine	one base mismatched with TBARW26C3	completely matched with TBARR26C5
4	1	BAR113iC			
5	1	Blank			
6	1	Blank			
7	1	BAR111iT	11mer + 4 inosine	completely matched with TBARW26C3	one base mismatched with TBARR26C5
8	1	BAR111iT			
9	1	Blank			
10	1	Blank			
11	1	BAR111iC	11mer + 4 inosine	one base mismatched with TBARW26C3	completely matched with TBARR26C5
12	1	BAR111iC			
13	1	Blank			
14	1	Blank			

Then, according to the protocol of TeleChem Inc., the oligonucleotide probe was immobilized on the microscope slide through terminal covalent bonds (a Schiff base was formed between amino residue of each oligonucleotide probe and aldehyde residue on slide surface) as follows. First, the microscope slide was mounted on a slide rack, and washed twice with 0.2% SDS at 25°C for 2 minutes with sufficient stirring in a beaker. Then, it was washed twice with sterilized water at 25°C for 2 minutes with sufficient stirring, and further treated with sterilized water at 98°C for 2 minutes. The microscope slide was air-dried at room temperature for 5 minutes, then transferred into a sodium hydrogenborate solution [prepared by dissolving 1 g of $NaBH_4$ in 300 ml of PBS buffer (prepared by dissolving 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogenphosphate, and 0.24 g of potassium dihydrogenphosphate in deionized water, adjusting it to pH 7.4 with hydrochloric acid, and filling it up to 1000 ml) and 100 ml of ethanol], treated in the solution at 25°C for 5 minutes, washed three times with 0.2% SDS at 25°C (room temperature) for 1 minute, finally washed with sterilized water at 25°C for 1 minute, and air-dried.

(3) Hybridization reaction

The Sso7d protein (to be 900 ng/ml in the final concentration) and 0.1 pmol of either the sample DNA Nos. (ix) or (8) were added to Annealing Buffer (buffer composition: 20 mM Tris buffer, pH 7.5, 2 mM DTT, 5 mM $MgCl_2$,

0.1 μg/μl BSA, 1 mM ATP (all showed in the final concentration)) to obtain hybridization solution. The hybridization solution (total volume: 15 μl) was applied to the oligonucleotide probe-immobilized microscope slide (DNA microarray) formed in the aforementioned step (2) by using hybridization seal (produced by Takara Shuzo Co.) to perform hybridization of the DNA samples Nos. (xi) or (8) at 60°C for 6 minutes in the solution. As a control, hybridization reaction was also performed in the same manner without adding the Sso7d protein.

After the hybridization reaction, the microscope slide was washed with 1 x SSC/0.03% SDS buffer at 25°C for 5 minutes, rinsed with 0.2 x SSC, and then further rinsed with 0.5 x SSC. After the washing solution was removed by centrifugation, the microscope slide was air-dried.

By using ScanArray 3000 of General Scanning Inc., wherein a Cy3 detecting channel (Ch.1) and a Cy5 detecting channel (Ch.2) were switched over as required, a hybridization image on microscope slide of each channel was obtained, and then the intensity of hybridization signal was evaluated by measuring the amount of fluorescence of each spot of the obtained hybridization image by using quantitative software, ImaGene ver.2 (produced by BioDiscovery Inc.).

The obtained hybridization image is shown in Figure 2. Cy3 detecting at Ch.1 (Figure 2, Upper two figures) and Cy5 detecting at Ch.2 (Figure 2, Lower two figures) were performed, and then each of hybridization under condition

without adding Sso7d protein (condition a of Figure 2) and under condition with adding Sso7d protein (condition b of Figure 2) was compared. The representative examples of data obtained by measuring intensity of hybridization signal of spot detected by Cy5 detecting are shown in below Table 5.

Table 5

Without adding Sso7d (shown in Figure 2 lower left)

Column	Row	Probes	Signal Strength(Average)
2	5,6	BAR113T(One-base mismatch)	1,000
2	9,10	BAR113C(Perfect match)	2,000
2	13,14	BAR113iT(One-base mismatch)	800
1	3,4	BAR113iC(Perfect match)	1,000
1	7,8	BAR111iT(One-base mismatch)	800
1	11,12	BAR113iC(Perfect match)	1,000
Background			200-2,000

With adding Sso7d(shown in Figure 2 lower right)

Column	Row	Probes	Signal Strength(Average)
2	5,6	BAR113T(One-base mismatch)	1,000
2	9,10	BAR113C(Perfect match)	60,000
2	13,14	BAR113iT(One-base mismatch)	1,000
1	3,4	BAR113iC(Perfect match)	60,000
1	7,8	BAR111iT(One-base mismatch)	400
1	11,12	BAR113iC(Perfect match)	1,000
Background			100-200

In the hybridization systems not added with Sso7d protein, it was found that the intensity of hybridization signal between the oligonucleotide probe immobilized on the substrate (on the DNA array) and the DNA sample was weak, background was high and hybridization level was uneven. When the S/N ratio was calculated by using background value, the strongest intensity was 10 (2000/200). However, most signal intensity was lower than several fold. The difference of the signal intensity between the case of using the completely matched probe and the case of using one base-mismatched probe was small.

On the other hand, when the Sso7d protein was added to the hybridization systems, it is found that the intensity of hybridization signal between the oligonucleotide probe immobilized on the substrate and the DNA sample is distinctly strong, background is depressed lower and hybridization level is even. When the S/N ratio was calculated by using background value, the stronger intensity was more than 300 (60000/200). The intensity of hybridization signal of completely matched probes definitely emitted stronger compared with that of one-base mismatched probes, which shows complete homology can be confirmed easily by the present invention.

That is, in hybridization between the oligonucleotide probe and the DNA sample on the DNA microarray, it was found that adding Sso7d protein makes the S/N ratio of hybridization signal higher, because of a considerable increase of hybridization signal intensity and decrease of background,

and have the effects of reducing unevenness of hybridization thereby the uniform hybridization reaction can be achieved.

As a result, reliability of the quantitative data obtained by this method is considerable high. According to the method of the present invention, gene analysis by using the DNA array or the DNA chip, on which either an oligonucleotide probe or a DNA sample is immobilized, can be performed with high speed and high sensitivity using a plurality of samples, which means the present invention can provide gene analysis in a high-throughput manner.

Remarks

I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of application or any patent issuing thereon.

Date: June 1st, 2001

Kazuhisa Hatakeyama

Kazuhisa HATAKEYAMA